Supporting Information

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Section 1. Dose–Response Curve for Multiple Reactions

Consider the sequence of reactions given by

$$R + S \leftrightarrow RS$$
$$RS + D \leftrightarrow RSD.$$

The concentration equations for RS and RSD are $[RS] = q_1[R][S]$ and $[RSD] = q_2[RS][D]$, whereas the mass-conservation equations are $[R] + [RS] + [RSD] = R^T$ and $[D] + [RSD] = D^T$. Combining these equations results in a quadratic (nonlinear) equation in $[RSD]$:

$$q_1q_2[S][RSD]^2 - (1 + q_1(1 + q_2(R^T + D^T))[S])[RSD] + q_1q_2R^TD^T[S] = 0, \quad \textbf{[S1.1]}$$

which can be solved to obtain

$$[RSD] = \frac{1 + q_1(1 + q_2(R^T + D^T))[S] - \sqrt{(1 + q_1(1 + q_2(R^T + D^T))[S])^2 - 4q_1^2q_2^2RTD^T[S]^2}}{2q_1q_2[S]}$$

and where the correct root is chosen such that $[RSD]$ is zero when $[S]$ is zero. Thus, $[RSD]$ does not have a first-order Hill dose–response curve (FHDC). We can demonstrate that $[RSD]$ does not even mimic an FHDC by calculating the fold change between 10 and 90% of maximal $[RSD]$. For an FHDC, this value is 81. We first define new parameters, so we can rewrite Eq. **S1.1** as

$$a[S][RSD]^2 - (1 + b[S])[RSD] + c[S] = 0. \quad \textbf{[S1.2]}$$

Define S_p to be the amount of steroid that induces $pY_{\max}$, where p is a fraction and the maximal induction $Y_{\max}$ is the solution of the critical-point equation $aY_{\max}^2 - bY_{\max} + c = 0$. Hence, $0 \le Y_{\max} \le b/2a$. Solving Eq. **S1.2** for $[S]$ gives

$$[S] = \frac{[RSD]}{a[RSD]^2 - b[RSD] + c}. \quad \textbf{[S1.3]}$$

Substituting in $[RSD] = pY_{\max}$ and $c = -aY_{\max}^2 + bY_{\max}$ in Eq. **S1.3** yields

$$S_p = \frac{p}{(p^2 - 1)aY_{\max} + (1-p)b}.$$

The p to $1 - p$ fold change is then

$$\frac{S_{1-p}}{S_p} = \left(\frac{1-p}{p}\right)^2 \frac{b - (1+p)aY_{\max}}{b - (2-p)aY_{\max}}.$$

Hence, the 10–90% fold change is given by $81(1 - 1.1z)/(1 - 1.9z)$, where $z = aY_{\max}/b$ and $0 \le z \le 0.5$. The fold change is an increasing function of $Y_{\max}$ and is not FHDC except when $Y_{\max}$ is small, which is also where the quadratic term in Eq. **S1.1** is small. This proof can be generalized to an arbitrary number of reactions. Dropping the quadratic term in Eq. **S1.1** results in an equation that is bilinear in $[S]$ and $[RSD]$, in which case $[RSD]$ has an FHDC.

Consider now a third reaction, $RSD + U \Leftrightarrow RSDU$. There is now an additional concentration equation $[RSDU] = q_3[RSD][U]$, and the conservation equations become $[R] + [RS] + [RSD] + [RSDU] = R^T$, $[D] + [RSD] + [RSDU] = D^T$, and $[U] + [RSDU] = U^T$. To derive an equation for $[RSDU]$ in terms of $[S]$, we eliminate the other variables recursively. We first combine $[RSD] = [RSDU]/q_3[U]$ with $[U] = [RSDU] - U^T$ to obtain $[RSD] = [RSDU]/q_3(U^T - [RSDU])$. Similarly, we use this result and the other equations to obtain

$$[RS] = \frac{[RSDU]}{q_2q_3(D^T - [RSDU])(U^T - [RSDU]) - [RSDU]}$$

and

$$[R] = \frac{[RSDU]}{q_1(q_2q_3(D^T - [RSDU])(U^T - [RSDU]) - [RSDU])[S]}.$$

Substituting into $[R] + [RS] + [RSD] + [RSDU] = R^T$ then gives a quartic equation in $[RSDU]$, which does not yield an FHDC.

Section 2. Derivation of the FHDC for the General Theory

For reactions obeying the concentration equations and conservation Eq. **3** in the main text, the equations can be solved in pairs so that each product is a first-order Hill function of the previous product, that is,

$$[Y_i]([Y_{i-1}]) = \frac{v_i[Y_{i-1}]}{1 + w_i[Y_{i-1}]}, \quad \textbf{[S2.1]}$$

where v_i and w_i have explicit formulas listed in Table 1 in the main text and have different forms depending on location with respect to the concentration-limiting step (CLS). We can then *compose* these first-order Hill functions (i.e., substitute one function into another) to obtain any downstream product as a function of any upstream product.

The calculation is simplified by the observation that the first-order Hill function is in the family of fractional linear or Möbius transformations and forms a group under function composition, which can be represented by matrix multiplication of 2-by-2 matrices. Consider a set of fractional linear functions written in the form of Eq. **S2.1**, and we want to compute the composition of two functions $[Y_2]([Y_1]([Y_0]))$ to obtain $[Y_2]$ as a function of $[Y_0]$. The coefficients of each function are entered as elements of a matrix $[Y_i] \Rightarrow \begin{pmatrix} v_i & 0 \\ w_i & 1 \end{pmatrix}$. Then $[Y_2]([Y_1]([Y_0]))$ is given by a multiplication of the matrices representing $[Y_2]$ and $[Y_1]$, that is, $\begin{pmatrix} v_2 & 0 \\ w_2 & 1 \end{pmatrix}\begin{pmatrix} v_1 & 0 \\ w_1 & 1 \end{pmatrix} = \begin{pmatrix} v_2v_1 & 0 \\ w_2v_1 + w_1 & 1 \end{pmatrix}$, from which the composed function can be reconstructed to obtain $[Y_2]([Y_1]([Y_0])) = \frac{v_2v_1[Y_0]}{1 + (w_2v_1 + w_1)[Y_0]}$. Using this matrix representation, we can easily calculate any product $[Y_m]$ as a fractional linear (first-order Hill) function of *any* previous product $[Y_{b-1}]$ $(b \le m)$ and obtain

$$[Y_m]([Y_{b-1}]) = \frac{V_b^m[Y_{b-1}]}{1 + W_b^m[Y_{b-1}]}, \quad \textbf{[S2.2]}$$

where $V_b^m = \prod_{i=b}^m v_i$, $W_b^m = \sum_{i=b}^m w_i \prod_{j=b}^{i-1} v_j$, with the convention $\prod_{i=a}^n x_i = 1$ if $n < a$ and $W_b^m = W_b^{cls}$, for $m \ge cls$. As is evident from comparison with Eq. **1** of the main text, $A_{\max} = V_b^m/W_b^m$ and $EC_{50} = 1/W_b^m$. For steps $m \ge cls$, the denominators of all products $[Y_m]$ are the same, so the sum of any number of products $[Y_{cls}]$ to $[Y_n]$ will maintain FHDC form. Thus, we can express the activity of the final protein product as $A = \sum_{m=cls}^n a_m[Y_m]$, where a_m are positive constants. Noting that

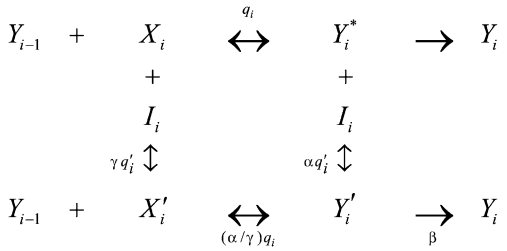
for $m \geq cls$, $[Y_m] = V_{cls}^m[Y_{cls}]$, using Eq. S2.2 we can rewrite the final gene activity as

$$A = \Gamma[Y_{cls}] = \frac{\Gamma V_b^{cls}[Y_{b-1}]}{1 + W_b^{cls}[Y_{b-1}]}, \qquad \textbf{[S2.3]}$$

where $\Gamma = \sum_{k=cls}^{n} a_{k-cls} V_{cls+1}^k$.

Section 3. Inhibition

Consider the general reaction scheme for an inhibitor I_i acting on an activator X_i.



The mass-conservation law is

$$[X_i] + \varepsilon_i[Y_i^*] + [X_i'] + [Y_i'] = X_i^T$$

and the concentration equations are [1, 2]

$$[Y_i'] = \alpha q_i'[I_i][Y_i^*]$$
$$[Y_i^*] = q_i[Y_{i-1}][X_i]$$
$$[X_i'] = \gamma q_i'[I_i][X_i].$$

Substituting the individual equilibrium conditions back into the mass-conservation equation gives $[X_i] + \varepsilon_i q_i[Y_{i-1}][X_i] + \gamma q_i'[I_i][X_i] + \alpha q_i q_i'[I_i][Y_{i-1}][X_i] = X_i^T$, which can be solved for $[X_i]$ and then substituted back into the concentration equation for $[Y_i^*]$ to yield

$$[Y_i^*] = \frac{q_i X_i^T [Y_{i-1}]}{1 + \gamma q_i'[I_i] + q_i(\varepsilon_i + \alpha q_i'[I_i])[Y_{i-1}]}.$$

The two products $[Y_i^*]$ and $[Y_i']$ can then be summed via $[Y_i] = [Y_i^*] + \beta[Y_i']$ to obtain a final expression of

$$[Y_i] = \frac{q_i X_i^T (1 + \alpha\beta q_i'[I_i])[Y_{i-1}]}{1 + \gamma q_i'[I_i] + q_i(\varepsilon_i + \alpha q_i'[I_i])[Y_{i-1}]} \equiv \frac{S[Y_{i-1}]}{U + T[Y_{i-1}]}. \qquad \textbf{[S3.1]}$$

If the inhibitor acts at or after the CLS of a reaction sequence, then the equations are modified. If the inhibitor acts at the CLS ($i = cls$), then the mass-conservation equation becomes $[X_{cls}] + \varepsilon_{cls}[Y_{cls}^*] + [X_{cls}'] + [Y_{cls}'] + \sum_{k=cls+1}^{n} \varepsilon_k[Y_k] = X_{cls}^T$, leading to Eq. S3.1 but with $T = q_{cls}\left(\sum_{k=cls}^{n} \varepsilon_k \prod_{j=cls+1}^{k} v_j + \alpha q_{cls}'[I_{cls}]\right)$. If the inhibitor acts after the CLS, then the mass-conservation equation is $[X_i] + [X_i'] = X_i^T$, which implies only linear competitive inhibition can occur leading to Eq. S3.1 with $\alpha_i = 0$, $\beta_i = 0$, and $\varepsilon_i = 0$. $[Y_i]$ can be put into the standard form of $[Y_i] = v_i[Y_{i-1}]/(1 + w_i[Y_{i-1}])$ by setting $v_i = S/U$ and $w_i = T/U$, giving (*i*) $v_i = q_i X_i^T (1 + \alpha\beta q_i'[I])/(1 + \gamma q_i'[I])$ and $w_i = q_i(\varepsilon_i + \alpha q_i'[I])/(1 + \gamma q_i'[I])$ if $0 < i < cls$, (*ii*) v_i is the same as (*i*) but $w_i = q_i\left(\sum_{k=i}^{n} \varepsilon_k \prod_{j=i+1}^{k} q_j X_j^T + \alpha q_i'[I]\right)/(1 + \gamma q_i'[I])$ if $i = cls$, and (*iii*) $v_i = q_i X_i^T/(1 + \gamma q_i'[I])$ and $w_i = 0$ [i.e., same as (*i*) with $\alpha_i = 0$, $\beta_i = 0$, $\varepsilon_i = 0$], if $i > cls$. These results are summarized in Table 1 of the main text.

Section 4. Isolating Total Concentration of Cofactors in the Product Function

Here we show how the model form can be explicitly constructed for an arbitrary number of factors to be visible with effective parameters, which can then be compared to data. We show examples for two activators and for an inhibitor and an activator. From main-text Eq. **4**, the general form for the final product is

$$[P] = \Gamma[Y_{cls}] = \frac{\Gamma V_1^{cls}[Y_0]}{1 + W_1^{cls}[Y_0]},$$

where $\Gamma = \sum_{k=cls}^{n} a_{k-cls} V_{cls+1}^k$, $V_b^m = \prod_{i=b}^{m} v_i$, $W_b^m = \sum_{i=b}^{m} w_i V_b^{i-1}$, and v_i and w_i differ depending on position with respect to the CLS and type of factor (see Table 1 of the main text). Now suppose we have two activators and the first activator acts before the CLS at step i and the second acts at step j, which can be before, at, or after the CLS. We want to make the total concentrations X_i^T and X_j^T visible in the final product. We do so by isolating factors of v_i and v_j, and $w_{cls} = q_{cls}\sum_{k=cls}^{n} \varepsilon_k \prod_{j=cls+1}^{k} v_j$. For the case $i < j < cls$, we can rewrite $V_1^{cls} = V_1^{i-1} v_i V_{i+1}^{j-1} v_j V_{j+1}^{cls} = q_i q_j V_{i+1}^{j-1} V_{j+1}^{cls} X_i^T X_j^T$. Using the decomposition rule $W_a^b = W_a^c + V_a^c W_{c+1}^b$, we obtain

$$\begin{aligned} W_1^{cls} &= W_1^i + V_1^i W_{i+1}^{cls} \\ &= W_1^i + V_1^i\left(W_{i+1}^j + V_{i+1}^j W_{j+1}^{cls}\right) \\ &= W_1^i + v_i V_1^{i-1}\left(W_{i+1}^j + v_j V_{i+1}^{j-1} W_{j+1}^{cls}\right) \\ &= W_1^i + q_i X_i^T V_1^{i-1}\left(W_{i+1}^j + q_j X_j^T V_{i+1}^{j-1} W_{j+1}^{cls}\right). \end{aligned}$$

Thus, we can write

$$[P] = \frac{(C_1 + C_2 X_j^T) X_i^T [Y_0]}{1 + (C_3 + C_4 X_i^T + C_5 X_i^T X_j^T)[Y_0]}, \qquad \textbf{[S4.1]}$$

where $C_1 = 0$, $C_2 = \Gamma q_i q_j V_i^{i-1} V_{i+1}^{j-1} V_{j+1}^{cls}$, $C_3 = W_1^i$, $C_4 = q_i V_1^{i-1} W_{i+1}^j$, and $C_5 = q_i q_j V_1^{i-1} V_{i+1}^{j-1} W_{j+1}^{cls}$ are effective parameters. Eq. S4.1 gives the final product concentration as a function of the total concentrations of the factors X_i^T and X_j^T. However, a given factor may have an endogenous component and an added exogenous component. If we are interested in obtaining the dependence of the final product on the added exogenous component only, then we divide the total concentration into two pieces. For example, suppose X_j^T is composed of an endogenous part $X_j^{T,e}$ and an added exogenous part $X_j^{T,a}$. If we are interested in how $[P]$ changes in response to the added part, then we can write $X_j^T = X_j^{T,e} + X_j^{T,a}$ and redefine the parameters to absorb $X_j^{T,e}$. Doing so results in Eq. S4.1 but with $C_1 = \Gamma q_i q_j V_i^{i-1} V_{i+1}^{j-1} V_{j+1}^{cls} X_j^{T,e}$, $C_4 = q_i V_1^{i-1} W_{i+1}^j + q_i q_j V_1^{i-1} V_{i+1}^{j-1} W_{j+1}^{cls} X_j^{T,e}$. Eq. S4.1 is Eq. **5** in the main text with $R^T = X_i^T$ and $U^T = X_j^{T,a}$.

For the case $i < j = cls$, everything is the same as before except that $W_1^{cls} = W_1^i + q_i X_i^T V_1^{i-1} W_{i+1}^{cls}$, giving $C_3 = W_1^i$ and $C_5 = 0$. Finally, for the case $i < cls < j$, $\Gamma = \sum_{k=cls}^{j-1} a_{k-cls} V_{cls+1}^k + q_j X_j^T V_{cls+1}^{j-1} \sum_{k=j}^{n} a_{k-cls} V_{j+1}^k$ and

$$\begin{aligned} W_1^{cls} &= W_1^{cls-1} + w_{cls} V_1^{cls-1} \\ &= W_1^i + q_i X_i^T V_1^{i-1} W_{i+1}^{cls-1} + q_i X_i^T V_1^{i-1} V_{i+1}^{cls-1} w_{cls} \\ &= W_1^i + q_i X_i^T V_1^{i-1} W_{i+1}^{cls-1} + q_i \varepsilon_{cls} X_i^T V_1^{i-1} V_{i+1}^{cls-1} \\ &\quad \times\left(\sum_{k=cls}^{j-1} \varepsilon_k V_{cls+1}^k + \sum_{k=j}^{n} \varepsilon_k V_{j+1}^k q_j X_j^T V_{cls+1}^{j-1}\right) \\ &= W_1^i + q_i X_i^T V_1^{i-1} W_{i+1}^{cls-1} + q_i q_{cls} X_i^T V_1^{i-1} V_{i+1}^{cls-1} \\ &\quad \times \sum_{k=cls}^{j-1} \varepsilon_k V_{cls+1}^k + q_i q_{cls} X_i^T V_1^{i-1} V_{i+1}^{cls-1} q_j X_j^T V_{cls}^{j-1} \sum_{k=j}^{n} \varepsilon_k V_{j+1}^k \end{aligned}$$

leading to $C_1 = q_i V_i^{i-1} V_{i+1}^{cls} \sum_{k=cls}^{j-1} a_{k-cls} V_{cls+1}^k$, $C_2 = q_i V_1^{i-1} V_i^{cls} q_j V_{cls+1}^{j-1} \sum_{k=j}^{n} a_{k-cls} V_{j+1}^k$, $C_3 = W_1^i$, $C_4 = q_i V_1^{i-1} W_{i+1}^{cls-1} + q_i V_1^{i-1}$

$V_{i+1}^{cls-1}\sum_{k=cls}^{j-1}\varepsilon_k V_{cls+1}^k$, and $C_5 = q_i q_j V_1^{i-1} V_{i+1}^{cls-1} V_{cls+1}^{j-1}\sum_{k=j}^{n}\varepsilon_k V_{j+1}^k$. The results for the three cases are summarized in Table S1.

Now, consider the action of an activator and inhibitor at step i. In this case, the activator and inhibitor could both act at the same step or at different steps. First consider the case where $i < cls$. Again we decompose

$$V_1^{cls} = V_1^{i-1} v_i V_{i+1}^{cls} = V_1^{i-1} V_{i+1}^{cls} \frac{q_i X_i^T (1 + \alpha_i \beta_i q_i'[I_i])}{1 + \gamma_i q_i'[I_i]}$$

and

$$\begin{aligned} W_1^{cls} &= W_1^{i-1} + V_1^{i-1} W_i^{cls} \\ &= W_1^{i-1} + V_1^{i-1}(w_i + v_i W_{i+1}^{cls}) \\ &= W_1^{i-1} + V_1^{i-1}\left(\frac{q_i(\varepsilon_i + \alpha_i q_i'[I_i])}{1 + \gamma_i q_i'[I_i]} + \frac{q_i X_i^T (1 + \alpha_i \beta_i q_i'[I_i])}{1 + \gamma_i q_i'[I_i]} W_{i+1}^{cls}\right) \end{aligned}$$

to obtain the final product

$$[P] = \frac{(D_0(1 + q_i'\gamma_i[I_i]) + D_1 X_i^T (1 + q_i'\alpha_i\beta_i[I_i]))[Y_0]}{\begin{aligned}&1 + q_i'\gamma[I_i] + (D_2(1 + q_i'\gamma_i[I_i]) \\ &+ D_3(D_4 + q_i'\alpha_i[I_i]) + D_5 X_i^T (1 + q_i'\alpha_i\beta_i[I_i]))[Y_0]\end{aligned}}, \quad \textbf{[S4.2]}$$

where $D_0 = 0, D_1 = \Gamma V_{i+1}^{cls} q_i V_1^{i-1}$, $D_2 = W_1^{i-1}$, $D_3 = q_i V_1^{i-1}$, $D_4 = \varepsilon_i$, and $D_5 = q_i V_1^{i-1} W_{i+1}^{cls}$. If necessary, we can break X_i^T into an endogenous and an exogenous part and then redefine the parameters as before. For the case $i = cls$,

$$W_1^{cls} = W_1^{cls-1} + V_1^{cls-1}\left(\frac{q_{cls}\left(\sum_{k=cls}^{n}\varepsilon_k V_{cls+1}^k + \alpha_i q_{cls}'[I_i]\right)}{1 + \gamma_i q_i'[I_i]}\right)$$

so that the D parameters are the same as for $i < cls$ except $D_4 = \sum_{k=cls}^{n}\varepsilon_k V_{cls+1}^k$ and $D_5 = 0$. Finally, for $i = j > cls$, we have

$$\Gamma = V_1^{cls}\sum_{k=cls+1}^{i-1} a_{k-cls} V_{cls+1}^k + q_i V_1^{i-1}\sum_{k=i}^{n} a_{k-cls} V_{i+1}^k \frac{X_i^T}{1 + \gamma_i q_i'[I_i]}$$

and

$$\begin{aligned} W_1^{cls} = W_1^{cls-1} &+ V_1^{cls-1}\left(\frac{q_{cls}\sum_{k=cls}^{i-1}\varepsilon_k V_{cls+1}^k + q_{cls} q_i V_{cls+1}^{i-1}\sum_{k=i}^{n}\varepsilon_k V_{i+1}^k X_i^T}{1 + \gamma_i q_i'[I_i]}\right), \end{aligned}$$

which leads to $D_0 = V_1^{cls}\sum_{k=cls+1}^{i-1} a_{k-cls} V_{cls+1}^k$, $D_1 = q_i V_1^{i-1}\sum_{k=i}^{n} a_{k-cls} V_{i+1}^k$, $D_2 = W_1^{cls-1} + q_{cls} V_1^{cls-1}\sum_{k=cls}^{i-1}\varepsilon_k V_{cls+1}^k$, $D_3 = 0$, $D_4 = 0$, $D_5 = q_{cls} q_i V_1^{cls-1} V_{cls+1}^{i-1}\sum_{k=i}^{n}\varepsilon_k V_{i+1}^k$, and $\alpha_i = 0$. The parameters are summarized in Table S1.

Section 5. Influence of Factors on EC_{50} and A_{max}

From Eq. S4.2, we find that

$$A_{max} = \frac{D_0(1 + q'\gamma[I]) + D_1 X^T (1 + q'\alpha\beta[I])}{D_2(1 + q'\gamma[I]) + D_3(D_4 + q'\alpha[I]) + D_5 X^T (1 + q'\alpha\beta[I])}$$

and

$$EC_{50} = \frac{1 + q_i'\gamma[I]}{D_2(1 + q'\gamma[I]) + D_3(D_4 + q'\alpha[I]) + D_5 X^T (1 + q'\alpha\beta[I])},$$

where the D parameters are listed in Table S1 and we have dropped the index subscript. We can consider how the activator X^T or inhibitor $[I]$ affects A_{max} and EC_{50} individually. The derivative of A_{max} and EC_{50} with respect to X^T while fixing $[I] = 0$ is

$$\frac{\partial A_{max}}{\partial X^T} = \frac{D_1 D_2 - D_0 D_5}{(D_2 + D_5 X^T)^2} \quad \textbf{[S5.1]}$$

$$\frac{\partial EC_{50}}{\partial X^T} = \frac{-D_5}{(D_2 + D_5 X^T)^2} \quad \textbf{[S5.2]}$$

and the derivative of A_{max} and EC_{50} with respect to $[I]$ while fixing $X^T = 1$ is

$$\begin{aligned} \frac{\partial A_{max}}{\partial [I]} = N[&D_0((D_3 D_4 + D_5)\gamma - (D_3\alpha + D_5\alpha\beta)) \\ &+ D_1(D_2\alpha\beta - (D_2\gamma + D_3\alpha))] \end{aligned} \quad \textbf{[S5.3]}$$

$$\frac{\partial EC_{50}}{\partial [I]} = N[(D_3 D_4 + D_5)\gamma - (D_3\alpha + D_5\alpha\beta)], \quad \textbf{[S5.4]}$$

where $N = q'/(D_2(1 + q'\gamma[I]) + D_3(D_4 + q'\alpha[I]) + D_5(1 + q'\alpha\beta[I]))^2$. Although the parameters are composed of an unknown number of constants associated with the reactions, they can be estimated directly from the data as shown in *SI Section 6* below. If the derivatives are positive, negative, or zero, then the respective quantity increases, decreases, or does not change. The values of the parameters will then determine the sign of the derivative. However, some predictions about the mechanisms and location of a cofactor with respect to the CLS can be made based on how A_{max} and EC_{50} move with the addition of the cofactor.

Eq. S5.1 shows that A_{max} will increase with the addition of an activator if $D_1 D_2 - D_0 D_5 > 0$. This is satisfied if $D_0 = 0$ or is small, which can hold if the activator acts before or at the CLS or the endogenous level of the activator is low. A_{max} will increase very minimally (i.e., not change) if $D_1 << D_2$ and $D_0 = 0$. This could occur if there are reactions downstream of the activator that are much "slower" (i.e., association constants and/or total factor concentrations are much smaller). If the activator acts after the CLS, then A_{max} can move in any direction depending on the sign of $D_1 D_2 - D_0 D_5$. From Table S1, we see that the magnitude of D_1 is controlled by products downstream of the activator and D_0 by those upstream. Thus, A_{max} can decrease if the downstream reactions contribute less to the final product than upstream reactions (i.e., are slower) and vice versa. A_{max} does not change if the two contributions balance each other, although this would require fine-tuning and thus be unlikely. In summary, an activator will generally increase A_{max}. However, if there are slow reactions downstream of the activator, then A_{max} can decrease. The interesting point is that there are situations where A_{max} can decrease with the addition of an activator.

For an activator, Eq. S5.2 shows that EC_{50} always decreases unless $D_5 = 0$ or if $D_5 << D_2$. From Table S1 we see that $D_5 = 0$ if an activator acts at the CLS. The inequality $D_5 << D_2$ could be satisfied if there are reactions following the activator that are slower. Hence, EC_{50} generally decreases if an activator acts before or after the CLS but could possibly change very slowly and appear not to change. If the activator acts at the CLS then EC_{50} will not change. Hence, an activator cannot increase EC_{50}.

From Eqs. S5.3 and S5.4, we see that determining the action of an inhibitor is less straightforward. However, we can still make some general statements if we consider the actions of different types of inhibition (i.e., competitive, uncompetitive, noncompetitive, linear, partial) acting before, at, or after the CLS. If the inhibitor acts before or at the CLS, then $D_0 = 0$ and A_{max} decreases for competitive ($\alpha = 0$) or linear inhibition ($\beta = 0$). If the inhibitor acts after the CLS, then there can only be competitive inhibition ($\alpha = 0$), which means $\partial A_{max}/\partial [I] = N\gamma [D_0 D_5 - D_1 D_2]$. Thus, A_{max} can increase if the downstream reactions contribute less to the final product than upstream ones and vice versa, and

does not change when the two are balanced. A_{max} can also not change very much for a competitive or linear inhibitor if $D_1 << D_2^{'}$. EC_{50} does not change (increases weakly, while A_{max} decreases) if the inhibitor acts after the CLS and $D_2 >> D_3D_4 + D_5$, which is possible if there are downstream reactions that are slower.

From Eq. **S5.4**, we see that EC_{50} decreases for uncompetitive inhibition ($\gamma = 0$) and increases for competitive inhibition ($\alpha = 0$). EC_{50} does not change for a partial noncompetitive inhibitor acting before the CLS ($D_0 = 0$, $D_4 = 1$, $\alpha = \gamma$, $\beta = 1$). These behaviors can still hold if the inhibition is mixed but close to these special cases. Thus, for inhibition, it is possible for A_{max} and EC_{50} to both move in any direction.

From these conditions, we can make some predictions of the actions of cofactors listed in Table 2 of the main text. We note that these predicted mechanisms are not exclusive. For specificity and brevity, we assumed the simplest possibility in Table 2. More precise predictions can only be made if the D parameters are estimated from the data.

Section 6. Model Fit to Data and Parameter Estimates for Ubc9 and Glucocorticoid Receptor

We fit the model for two activators [glucocorticoid receptor (R^T) and Ubc9 (U^T)] and steroid (S^T) (dexamethasone) given by Eq. 5 of main text and Eq. **S4.1** to experimental data. The dataset consisted of luciferase activity as a function of S^T, R^T, and U^T. In all there were 60 data points taken each in triplicate (see *SI Section 8*). The actual form of the model used for the fits was

$$A = \frac{\alpha K_1 K_2 D^T R^T (K_3 + K_4 U^T)\, S^T}{1 + K_1(1 + K_2 R^T (1 + K_3 + K_4 U^T)) S^T},$$

where A is the luciferase activity, and the concentrations of S^T, R^T, and U^T are nanomolar. We then fit to the K parameters and α. D^T was an extraneous free parameter that was fixed to an arbitrary number. The proportionality constant α represents the luminosity per mole of output protein luciferase and the proportionality constant of luciferase to the amount of final complex. This form is just a reparameterization of Eq. **5** in the main text.

The experiments use plasmids for glucocorticoid receptor (GR) and Ubc9. To fit the model to the data, we assumed that the amount of protein expressed is proportional to the amount of plasmids added. The actual proportionality constant is not important for the fits, but for convenience we estimated the concentrations of protein expressed based on the amount of added plasmids. Assuming 660 g/mol per base pair, a well volume of 0.00033 L, and 6800 bp per pSG5/GR plasmid and 5163 bp/Ubc9 plasmid, we calculated the concentration of plasmid (nM) from the mass in ng of plasmid added. We assumed that the concentration of the actual number of proteins translated is directly proportional to the concentration of the plasmid. From these estimates, we equated 0.1, 2, 10, and 25 ng of GR plasmid with 6.75E-5, 0.00135, 0.00675, and 0.0169 nM GR, and 135 and 175 ng of Ubc9 plasmid with 0.12 and 0.16 nM. Again, these values are not important for the model fits. They only change the scale of the parameters. The only assumption that is important is that the amount of plasmid added is proportional to the amount of protein expressed in the cells.

The data were fit using a Bayesian Markov-chain Monte Carlo method, specifically a variant of the Metropolis–Hastings algorithm with parallel tempering [3]. Initial priors for the parameters $\{K_1, K_2, K_3, K_4, \alpha, D_T\}$ were {0.04285, 1335, 0.346736, 3.549, 101,084, 0.080007} and bounded to the range {{0, 100}, {0, ∞}, {0, ∞}, {0, ∞}, {0, ∞}, {0, 0.080007}} with guess ranges of {1, 10, 30, 30, 100,000, 0.080007}. The upper bound of D^T was determined from the concentration of luciferase plasmid and was forced to be below that value. These guess ranges were determined empirically by trial and error to give a reasonable acceptance rate of the algorithm. The parallel tempering was run at different inverse "temperatures" (β) of 0.00001, 0.001, 0.1, 0.4, 0.7, and 1. The Monte Carlo algorithm was run for 100,000 iterations at each value of β and the first half of the results was discarded, resulting in 77.2%, 75.4%, 18.2%, 16.2%, 15.9%, and 15.6% acceptance rates, respectively. χ^2 values at each β were calculated using the second half of the trial fits (i.e., the last 50,000 values) to allow the transients to decay. The log likelihood versus β was integrated to obtain the true χ^2 of −2066.27 for this model. The resulting equations for the maximum-likelihood parameters (lowest χ^2) are shown in Fig. 2 of the main text. The maximum-likelihood parameters had a χ^2 value of 1741.36 with parameter values {0.0390839, 1484.67, 0.376871, 4.45226, 183930, 0.0414917}. The posterior statistics of the parameters are in Table S2.

By the telescoping property of the FHDC, the predicted luciferase activity for Ubc9 acting after the CLS is consistent with either a "hit-and-run" scheme

$$\begin{aligned} S + R &\Leftrightarrow RS \\ RS + D &\Leftrightarrow RS + D^{'} \\ D^{'} + U &\Leftrightarrow D^{*} + U \\ A &= \alpha[D^{'}] + \beta[D^{*}] \end{aligned}$$

or a complex-forming scheme under the concentration-limiting conditions predicted by Eq. **3** of the main text,

$$\begin{aligned} S + R &\Leftrightarrow RS \\ RS + D &\Leftrightarrow RSD \\ RSD + U &\Leftrightarrow RSDU \\ A &= \alpha[RSD] + \beta[RSDU], \end{aligned}$$

where U is Ubc9 and D is an unspecified cofactor acting at the CLS. As an independent test, we also fit the prediction generated by the complex-forming scheme (i.e., not necessarily obeying the conservation equations that preserve FHDC), which requires the solution of a nonlinear equation in $[RSD]$ and $[RSDU]$. We found that this scheme could fit the data only if the parameters have values such that the conservation equation has the form of Eq. **3** for which the activity is the same as for the hit-and-run scheme (i.e., Eq. **5** in the main text), thus validating the uniqueness of the model.

Section 7. GR Dimerization and FHDC

We show that GR dimerization cannot be necessary for steroid-induced gene induction to have an FHDC. Consider the first four reactions of a reaction sequence that includes dimerization

$$\begin{aligned} Y_0 + X_1 &\Leftrightarrow Y_1 \\ Y_1 + Y_1 &\Leftrightarrow Y_{1D} \\ Y_1 + X_2 &\Leftrightarrow Y_2^{*} \\ Y_{1D} + X_2 &\Leftrightarrow Y_2^{'} \end{aligned}$$

and Y_2^{*} and $Y_2^{'}$ both enter subsequent reactions. The concentration equations for the first two reactions are

$$\begin{aligned} [Y_1] &= q_1[X_1][Y_0] \\ [Y_{1D}] &= q_{1D}[Y_1]^2 \end{aligned}$$

and the conservation equation is

$$[X_1] + [Y_1] + 2[Y_{1D}] = X_1^T.$$

Substituting for $[Y_1]$ and $[Y_{1D}]$ from the concentration equations into the conservation equation leads to

$$[X_1] + q_1[X_1][Y_0] + 2q_{1D}q_1^2[X_1]^2[Y_0]^2 = X_1^T.$$

This quadratic equation can be solved to yield $[X_1] = \frac{-(1+q_1[Y_0])+\sqrt{(1+q_1[Y_0])^2+8q_{1D}q_1^2[Y_0]^2X_1^T}}{4q_{1D}q_1^2[Y_0]^2}$, which gives $[X_1] \approx X_1^T/1 + q_1[Y_0]$ if $8q_{1D}q_1^2[Y_0]^2X_1^T << (1+q_1[Y_0])^2$ leading to

$$[Y_1] \approx \frac{q_1X_1^T[Y_0]}{1+q_1[Y_0]}, [Y_{1D}] \approx q_{1D}\left(\frac{q_1X_1^T[Y_0]}{1+q_1[Y_0]}\right)^2,$$

and so $[Y_{1D}]$ is not a first-order Hill function. Now, consider the downstream reactions $Y_1 + X_2 \Leftrightarrow Y_2^*$ and $Y_{1D} + X_2 \Leftrightarrow Y_2'$ with concentration equations

$$[Y_2^*] = q_2^*[X_2][Y_1]$$
$$[Y_2'] = q_2'[X_2][Y_{1D}]$$

and conservation equation $[X_2] + [Y_2'] + [Y_2^*] = X_2^T$. Substituting for $[Y_2^*]$ and $[Y_2']$ from the concentration equations into the mass-conservation equations gives $[X_2] + q_2[X_2][Y_1] + q_2^*[X_2][Y_{1D}] = X_2^T$, which can be solved for $[X_2]$ and substituted back into the concentration equations for $[Y_2^*]$ and $[Y_2']$. If we assume that $[Y_2] = a[Y_2'] + b[Y_2^*]$, then

$$[Y_2] = \frac{\left(aq_2^*[Y_1] + bq_2'[Y_{1D}]\right)X_2^T}{1 + q_2^*[Y_1] + q_2'[Y_{1D}]}$$
$$= \frac{(aq_2^*q_1X_1^T[Y_0](1+q_1[Y_0]) + bq_{1D}q_2'q_1^2(X_1^T)^2[Y_0]^2)X_2^T}{(1+q_1[Y_0])^2 + q_2^*q_1X_1^T[Y_0](1+q_1[Y_0]) + q_2'q_{1D}q_1^2(X_1^T)^2[Y_0]^2},$$

from which we see that FHDC can occur with dimerization only if $aq_2^* >> \frac{bq_{1D}q_2'q_1X_1^T[Y_0]}{1+q_1[Y_0]}$.

This implies that either the dimer concentration or the downstream effect of the dimer must be small.

Section 8. Experimental Procedures

Unless otherwise indicated, all operations were performed at 0 °C.

Chemicals. Dexamethasone (Dex) is from Sigma. The dual-luciferase reporter assay is from Promega.

Plasmids. Rat GR (pSG5-GR), GREtkLUC, and TIF2/GRIP1 [4] and pSG5/Ubc9 [5] have been described. Wild-type and mutant rat GRs (A477T, R479D, and D481R in pCMV4 neo) were generous gifts from David Pearce (University of California, San Francisco, CA). The double mutant (rGRA477T/I646A) was created by following the manufacturer's protocol for the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit to introduce a point mutation at amino acid 646 into rGR-A477T/pCMVneo using the following primers (mutant nucleotides are underlined): rGRI646A forward (5′-CTCTGCTTTGCTCCTGATCTGGCTATTAATGAGCAGAGAATGTC-3′), rGRI646A reverse (5′-GACATTCTCTGCTCATTAATAGCCAGATCAGGAGCAAAGCAGAG-3′). All mutations were verified by sequencing of the entire receptor gene, during which it was discovered that three receptors (A477T, R479D, and A477T/I646A) contained 9 additional glutamine residues (for a total of 28) in the polyglutamine repeat starting at position 78. This is not unusual, as polymorphisms of rat GR have been noted before and synthetic GRs with up to 80 glutamines in this region have been found to display no marked differences in protein expression, steroid binding, or transactivation [6–8]. A477T/I646A without the additional 9 glutamine residues was prepared by removing the LguI/BstXI fragment from the initially prepared plasmid and inserting it into LguI/BstXI-digested pSG5/GR. This construct was used in the experiments of Fig. 2 and Fig. S3.

Cell Culture, Transient Transfection, Reporter Analysis, and Western Blotting. Triplicate samples of cells were transiently transfected, assayed for luciferase activity, analyzed, and plotted as before, as was western blotting [4, 9]. Best fits of the dose–response curves (R^2 almost always > 0.95) following first-order Hill plots were obtained with KaleidaGraph (Synergy Software) unless otherwise specified.

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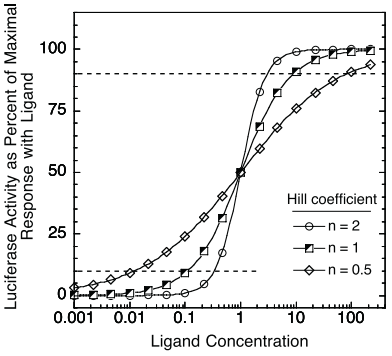

Fig. S1. Different possible shapes of sigmoidal dose–response curves. Dose–response curves with different values of the Hill coefficient *n* are shown. The dashed lines indicate the change in ligand concentration needed to produce the same response for curves with different values of *n*.

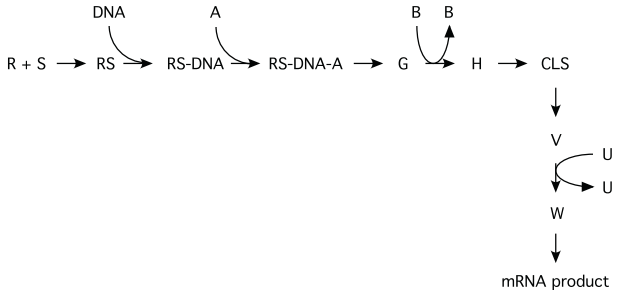

Fig. S2. Examples of some possible steps in steroid hormone action that can result in an FHDC. For simplicity, not all currently proposed steps are shown and no attempt has been made to identify which steps are reversible/cycling or irreversible, because all are allowed as long as the reactions reach a stationary or steady state. The steroid (S) binds to receptor (R), which then binds to DNA. The bound RS-DNA complex must have a smaller average concentration than the RS complex, or interact transiently. If a cofactor A binds to the RS-DNA complex, then it must also act transiently or its average concentration must be small compared with the concentration of the bound RS-DNA complex. The bound RS-DNA-A complex can transition directly into another state G. State G can be induced in a "hit-and-run" reaction by a cofactor B to transition into a state H. Some point in the reaction sequence could contain a CLS step. After the CLS, the concentrations of the free cofactors are unperturbed by reactions. Each of the steps after the CLS can be combined to form the final mRNA product, which is then translated into protein.

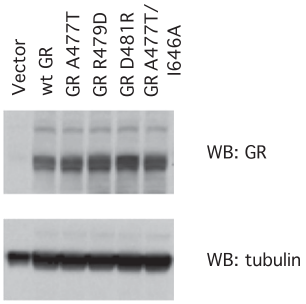

Fig. S3. Western blots of overexpressed GR mutant proteins in Cos-7 cells. Cell lysates were separated on 5–12% SDS/PAGE gels and visualized by western blotting with anti-GR antibody (Affinity Bioreagents; PA1-512). The equal amounts of tubulin (anti-tubulin antibody; AbCam ab4071-100) indicate that equal amounts of cell-lysate protein were used in each lane.

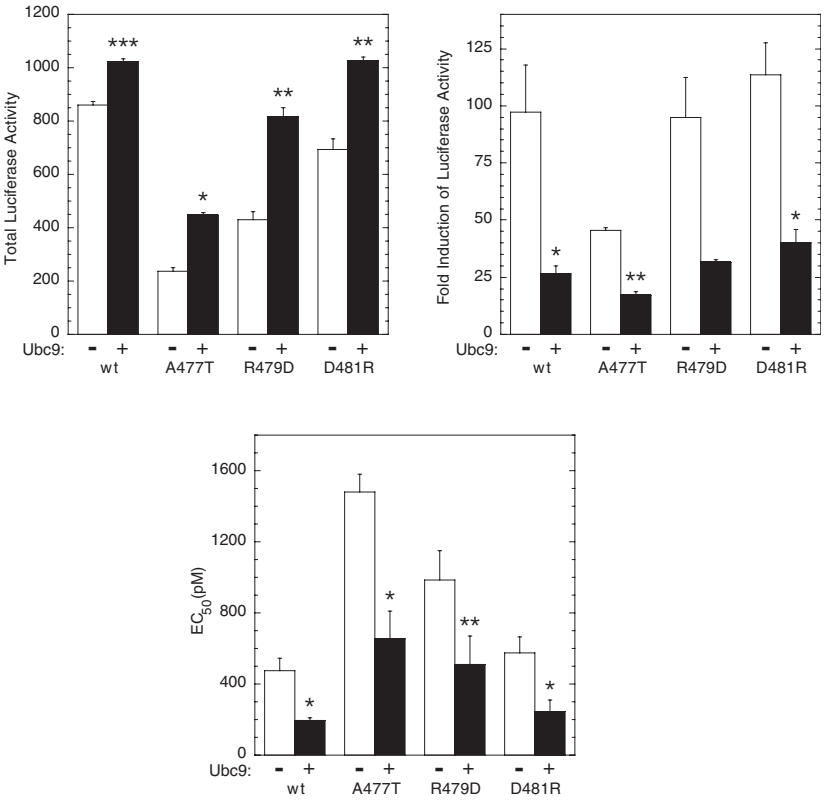

Fig. S4. Modulatory activity of Ubc9 is maintained with dimerization-defective GR mutants. The induction properties with Dex of CV-1 cells transiently transfected with GREtkLUC reporter and wild-type or single mutant GR (n = 3 for each panel) plasmids ± Ubc9 plasmid were determined as in Fig. 2. The average values (±SEM) were plotted. *$P < 0.05$, **$P \leq 0.005$, ***$P \leq 0.0005$ versus no Ubc9.

Table S1. Parameter values

Position	Two activators	Activator with inhibitor at same position ($i = j$)
Before CLS $i < cls$	$C_1 = 0$ or $C_1 = \Gamma q_i q_j V_i^{i-1} V_{i+1}^{j-1} V_{j+1}^{cls} X_j^{T,e}$	$D_0 = 0$,
	$C_2 = \Gamma q_i q_j V_i^{i-1} V_{i+1}^{j-1} V_{j+1}^{cls}$	$D_1 = \Gamma V_{i+1}^{cls} q_i V_1^{i-1}$
	$C_3 = W_1^i$	$D_2 = W_1^{i-1}$
	$C_4 = q_i V_1^{i-1} W_{i+1}^j$ or	$D_3 = q_i V_1^{i-1}$
	$C_4 = q_i V_1^{i-1} W_{i+1}^j + q_i q_j V_1^{i-1} V_{i+1}^{j-1} W_{j+1}^{cls} X_j^{T,e}$	$D_4 = \varepsilon_i$
	$C_5 = q_i q_j V_1^{i-1} V_{i+1}^{j-1} W_{j+1}^{cls}$	$D_5 = q_i V_1^{i-1} W_{i+1}^{cls}$
At CLS $i = cls$	Same as before CLS except $C_3 = W_1^i$ $C_5 = 0$	Same as before CLS except $D_4 = \sum_{k=cls}^{n} \varepsilon_k V_{cls+1}^k$ and $D_5 = 0$
After CLS $i > cls$	$C_1 = q_i V_i^{i-1} V_{i+1}^{cls} \sum_{k=cls}^{j-1} a_{k-cls} V_{cls+1}^k$	$D_0 = V_1^{cls} \sum_{k=cls+1}^{i-1} a_{k-cls} V_{cls+1}^k$
	$C_2 = q_i V_1^{i-1} V_i^{cls} q_j V_{cls+1}^{j-1} \sum_{k=j}^{n} a_{k-cls} V_{j+1}^k$	$D_1 = q_i V_1^{i-1} \sum_{k=i}^{n} a_{k-cls} V_{i+1}^k$
	$C_3 = W_1^i$	$D_2 = W_1^{cls-1} + q_{cls} V_1^{cls-1} \sum_{k=cls}^{i-1} \varepsilon_k V_{cls+1}^k$
	$C_4 = q_i V_1^{i-1} W_{i+1}^{cls-1} + q_i V_1^{i-1} V_{i+1}^{cls-1} \sum_{k=cls}^{j-1} \varepsilon_k V_{cls+1}^k$	$D_3 = 0$
	$C_5 = q_i q_j V_1^{i-1} V_{i+1}^{cls-1} V_{cls+1}^{j-1} \sum_{k=j}^{n} \varepsilon_k V_{j+1}^k$	$D_4 = 0$ $D_5 = q_{cls} q_i V_1^{cls-1} V_{cls+1}^{i-1} \sum_{k=i}^{n} \varepsilon_k V_{i+1}^k$ $\alpha_i = 0$

Table S2. Posterior statistics of parameters

Parameter	Mean	Median	SD	Interquartile range	Value	Maximum-likelihood value
K_1	0.041	0.040	0.001	0.00076	0.041 ± 0.001	0.039
K_2	1435	1430	60	75.6	1440 ± 60	1485
K_3	0.374	0.377	0.007	0.007	0.374 ± 0.007	0.377
K_4	4.5	4.5	0.4	0.5	4.5 ± 0.4	4.5
α	150,807	160,449	35,508	64,995	150,000 ± 40,000	183,930
Dt	0.054	0.047	0.01	0.02	0.05 ± 0.01	0.041